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Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.2009.06915.x

Molecular Microbiology (2009) 74(5), 1031–1043 ■
doi:10.1111/j.1365-2958.2009.06915.x
First published online 9 November 2009

Cytochrome P450 125 (CYP125) catalyses C26-hydroxylation to initiate sterol side-chain degradation in *Rhodococcus jostii* RHA1

Kamila Z. Rosłoniec,[1†] Maarten H. Wilbrink,[1†] Jenna K. Capyk,[2] William W. Mohn,[2] Martin Ostendorf,[3] Robert van der Geize,[1*] Lubbert Dijkhuizen[1] and Lindsay D. Eltis[2]

[1]*Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, PO Box 14, 9750 AA Haren, the Netherlands.*

[2]*Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, V6T 1Z3, Canada.*

[3]*Schering-Plough, PO Box 20, 5340 BH, Oss, the Netherlands.*

Summary

The *cyp125* gene of *Rhodococcus jostii* RHA1 was previously found to be highly upregulated during growth on cholesterol and the orthologue in *Mycobacterium tuberculosis (rv3545c)* has been implicated in pathogenesis. Here we show that *cyp125* is essential for *R. jostii* RHA1 to grow on 3-hydroxysterols such as cholesterol, but not on 3-oxo sterol derivatives, and that CYP125 performs an obligate first step in cholesterol degradation. The involvement of *cyp125* in sterol side-chain degradation was confirmed by disrupting the homologous gene in *Rhodococcus rhodochrous* RG32, a strain that selectively degrades the cholesterol side-chain. The RG32Ω*cyp125* mutant failed to transform the side-chain of cholesterol, but degraded that of 5-cholestene-26-oic acid-3β-ol, a cholesterol catabolite. Spectral analysis revealed that while purified ferric $CYP125_{RHA1}$ was < 10% in the low-spin state, cholesterol ($K_D^{app} = 0.20 \pm 0.08$ μM), 5α-cholestanol ($K_D^{app} = 0.15 \pm 0.03$ μM) and 4-cholestene-3-one ($K_D^{app} = 0.20 \pm 0.03$ μM) further reduced the low spin character of the haem iron consistent with substrate binding. Our data indicate that CYP125 is involved in steroid C26-carboxylic acid formation, catalysing the oxidation of C26 either to the corresponding carboxylic acid or to an intermediate state.

Accepted 5 October, 2009. *For correspondence. E-mail R.van.der.Geize@rug.nl; Tel. (+31) 50 3632257; Fax (+31) 50 3632154. †These authors contributed equally to this work.

Introduction

Cytochromes P450 (P450s) are a widely distributed class of haem-containing monooxygenases that are present in all domains of life. Their essential roles in diverse metabolic pathways have also generated considerable interest for their use as biocatalysts (Julsing *et al.*, 2008). Genome sequence data analysis has revealed that actinobacteria posses a remarkable number of genes encoding P450s compared with other prokaryotes (Lamb *et al.*, 2006; McLean *et al.*, 2006). For example, *Rhodococcus jostii* RHA1 harbours 29 genes predicted to encode P450s (McLeod *et al.*, 2006). While the biological function of most of these monooxygenases is still unknown, several of them have been implicated in sterol/steroid catabolism.

The microbial degradation of cholesterol (5-cholestene-3β-ol; Fig. 1, compound I) involves two processes: sterol side-chain elimination and steroid ring opening (Van der Geize and Dijkhuizen, 2004). The order of these two processes *in vivo* is unknown and may vary between microorganisms. Generally, oxidation of the cholesterol 3β-hydroxyl moiety and isomerization of Δ^5 into Δ^4 is thought to initiate sterol degradation (Sojo *et al.*, 1997; Chen *et al.*, 2006; Chiang *et al.*, 2008). This transformation is catalysed by either cholesterol oxidase (CHO; MacLachlan *et al.*, 2000) or 3β-hydroxysteroid dehydrogenase (3β-HSD; Yang *et al.*, 2007) and results in the formation of 4-cholestene-3-one (Fig. 1, compound II). Further degradation of 4-cholestene-3-one proceeds via hydroxylation at C26 to initiate side-chain degradation or oxidation of rings A and B analogous to ring degradation of 4-androstene-3,17-dione, resulting in the formation of 2-hydroxyhexa-2,4-diene-oic acid (Fig. 1, compound VI; Van der Geize *et al.*, 2007). Microbial sterol side-chain degradation has been studied at the biochemical level in more detail in *Nocardia* species and *Mycobacterium* sp. strains NRRL B-3683 and NRRL B-3805 (Sih *et al.*, 1968a,b; Marsheck *et al.*, 1972; Fujimoto *et al.*, 1982a,b). The latter two are capable of selectively degrading the 17-alkyl side-chains of cholesterol and phytosterols.

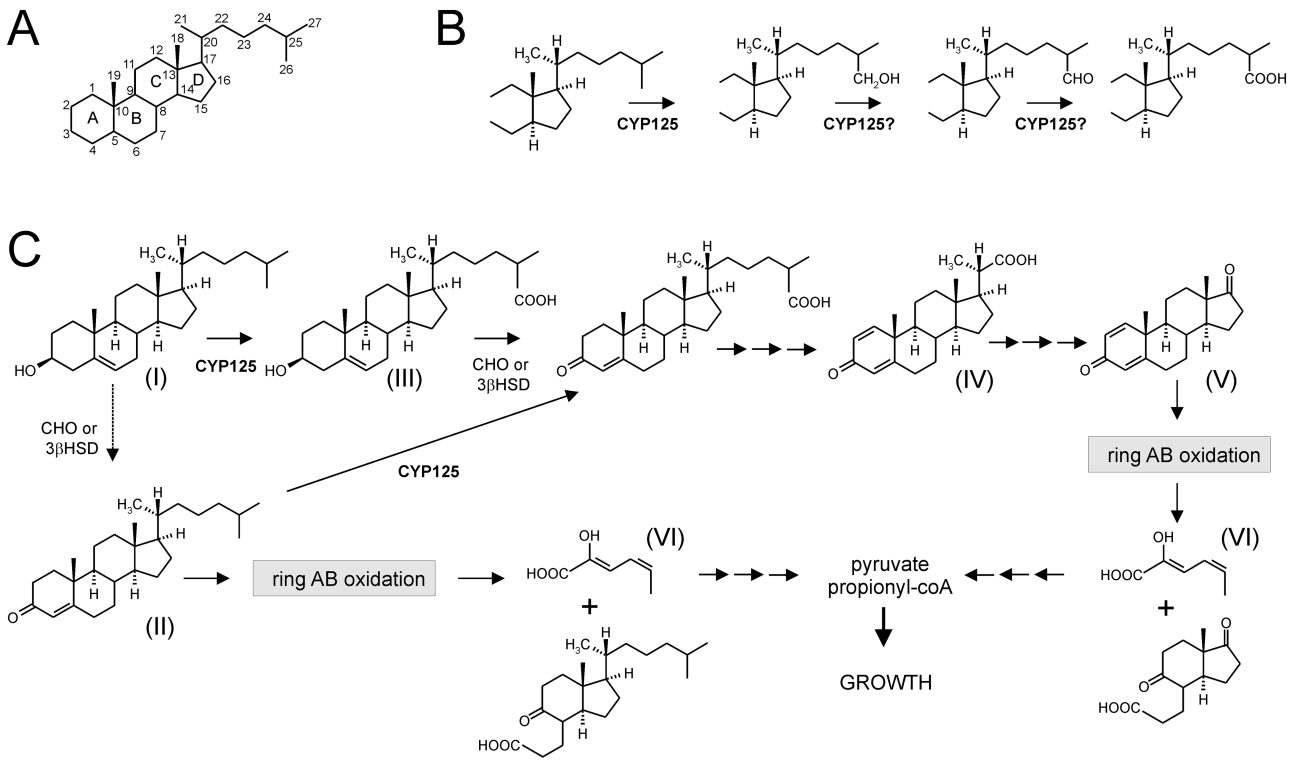


Fig. 1. The initial steps of aerobic cholesterol degradation in bacteria (Sih *et al*., 1968a,b; Szentirmai, 1990; van der Geize *et al*., 2007).
A. Steroid nomenclature.
B. CYP125 is involved in steroid C26 hydroxylation. Subsequent oxidation leads to a C26-oic acid metabolite.
C. Sterol degradation proceeds via steroid ring oxidation and side-chain degradation (upper route). The exact order of side-chain degradation and ring oxidation *in vivo* is unknown and may vary between microorganisms. In *R. jostii* RHA1, ring oxidation is not initiated until sometime after the side-chain attack by CYP125 (dotted arrow). The depicted metabolites are: (I) 5-cholestene-3β-ol (cholesterol), (II) 4-cholestene-3-one, (III) 5-cholestene-26-oic acid-3β-ol, (IV) 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid ($\Delta^{1,4}$-BNC), (V) 1,4-androstadiene-3,17-dione and (VI) 2-hydroxyhexa-2,4-diene-oic acid. *R. rhodochrous* mutant strain RG32 (see text) converts compound I into compounds IV and V by selective side-chain degradation. Abbreviations: CYP125, steroid 26-monooxygenase; CHO, cholesterol oxidase; 3βHSD, 3β-hydroxysteroid dehydrogenase (Yang *et al*., 2007).

Microbial cholesterol side-chain degradation is initiated by C26 hydroxylation followed by further oxidation to the sterol C26-oic acid (Fig. 1, compound III). Subsequent degradation occurs via a mechanism similar to β-oxidation of fatty acids that leads to the formation of a steroid C22-oic acid intermediate (Fig. 1, compound IV) with the concomitant release of propionyl-CoA and acetyl-CoA. The remaining C3 side-chain is released as propionyl-CoA via a different mechanism (Sih *et al*., 1967; 1968b).

Rhodococcus rhodochrous DSM43269 (synonym IFO3338) is able to selectively degrade the sterol side-chain in the presence of iron chelators, which inhibit 3-ketosteroid 9α-hydroxylase (KSH) activity (Arima *et al*., 1978). This phenotype was replicated in a stable multiple gene deletion mutant strain of *R. rhodochrous* DSM43269 (strain RG32) lacking KSH activity (M.H. Wilbrink, L. Dijkhuizen and R. van der Geize, unpublished). Mutant strain RG32 is completely blocked in steroid ring degradation and capable of selective sterol side-chain degradation, thereby accumulating 1,4-androstadiene-3,17-dione (ADD) (Fig. 1, compound V) and 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid ($\Delta^{1,4}$-BNC) (Fig. 1, compound IV) from sterols. The strain RG32 phenotype thus allows us to specifically analyse sterol side-chain degradation.

To date, genes involved in sterol side-chain degradation have not been identified. Using transcriptomic analysis, we recently identified a cholesterol catabolic gene cluster in *R. jostii* RHA1 that includes two P450-encoding genes (Van der Geize *et al*., 2007). Interestingly, *ro04679* (*cyp125*$_{RHA1}$) was one of the most highly upregulated genes within this cluster during growth on cholesterol, suggesting an important role for this enzyme in cholesterol catabolism. In the RHA1 genome, *cyp125* is located proximal to genes predicted to encode β-oxidation enzymes, and suggested to be involved in degradation of the alkyl side-chain of cholesterol (Van der Geize *et al*., 2007). Moreover, *cyp125* is located within the *ro04482-ro04705* gene cluster encompassing the *mce4* genes, which encode the uptake system for cholesterol and related steroids with unsubstituted alkyl side-chains (Mohn *et al*., 2008).

Here we report the molecular characterization of CYP125 as a steroid 26-monooxygenase. The *cyp125* gene was inactivated in each of *R. jostii* RHA1 and *R. rhodochrous* RG32 and the effect on cholesterol catabolism was elucidated. $CYP125_{RHA1}$ was heterologously expressed and purified, and its binding to cholesterol and its analogues was investigated. This study provides novel insights into bacterial steroid degradation, revealing that degradation in *R. jostii* RHA1 is initiated by side-chain oxidation, not oxidation of the rings.

Results

CYP125 possesses conserved amino acid residues for interaction with sterols

Bioinformatic analysis revealed that $CYP125_{RHA1}$ has high amino acid sequence identity with P450s from other actinobacteria, including *Nocardia farcinica* strain IFM10152 [Nfa5180, 79% (Ishikawa *et al*., 2004)] and *Mycobacterium tuberculosis* strain H37Rv [Rv3545c, 69% (Cole *et al*., 1998; Camus *et al*., 2002)]. These proteins belong to the uncharacterized CYP125 family (subfamily A) of P450 enzymes (Nelson *et al*., 1996), in which $CYP125_{RHA1}$ has been assigned CYP125A14P (http://drnelson.utmem.edu/biblioE.html#125). These monooxygenases presumably transform lipid-like compounds, as the CYP125 family includes many actinobacterial proteins associated with lipid degradation (Ventura *et al*., 2007).

Bioinformatic analysis further revealed that the annotated sequence of $CYP125_{RHA1}$ was about 50 residues longer than that of the annotated orthologues. Careful analysis of the $cyp125_{RHA1}$ nucleotide sequence indicated that the start codon most likely is located 159 nucleotides downstream from that in the original annotation, and is preceded by a Shine–Dalgarno sequence (aggag). Thus, $cyp125_{RHA1}$ is a gene of 1257 nucleotides, encoding a protein of 418 amino acids with a calculated molecular mass of 47.2 kDa. The re-annotated sequence of $cyp125_{RHA1}$ (RHA1 genome co-ordinates 4930900 . . . 4932156) was used in this study.

Amino acid sequence alignments revealed that the actinobacterial CYP125s share the conserved motifs characteristic for the P450 super-family, as well as key residues of cholesterol-transforming eukaryotic P450s (Fig. S1). The latter belong to various families, including: CYP3A4, which performs 4β-hydroxylation of cholesterol; CYP11A1, which transforms cholesterol to pregnenolone via C20–C22 bond-cleavage; CYP27A1, which hydroxylates cholesterol at C27; and CYP46A1, which transforms cholesterol to 24*S*-hydroxycholesterol (Mast *et al*., 2006; Pikuleva, 2006; Storbeck *et al*., 2007). The presence of these conserved residues in CYPs125 and in P450 enzymes known to interact with sterols suggests that sterols are substrates for CYP125.

Table 1. Growth in mineral media supplemented with cholesterol (2.5 mM) as sole carbon and energy source of wild-type strain RHA1, mutant strain RHA1Δ*cyp125*, complemented mutant strain RHA1Δ*cyp125* (RHA1Δ*cyp125*+pTip-QC1*cyp125*), and RHA1Δ*cyp125* mutant strain harbouring null vector (RHA1Δ*cyp125*+pTip-QC1) after 10 days of growth.

Strain	Protein content (mg l^{-1})	Residual cholesterol (%)
RHA1	49 ± 6	56 ± 5
RHA1Δ*cyp125*	5 ± 3	112 ± 4
RHA1Δ*cyp125*+pTip-QC1*cyp125*	57 ± 4	55 ± 4
RHA1Δ*cyp125*+pTip-QC1	3 ± 3	116 ± 16
Control (medium + cholesterol)	0	100 ± 13

Non-inoculated mineral medium with cholesterol was included as a negative control. Values represent mean ± standard deviation ($n = 3$).

CYP125 is essential for growth on 3-hydroxy-sterols

To elucidate the role of *cyp125* in sterol/steroid catabolism, an unmarked single gene deletion mutant strain, RHA1Δ*cyp125*, was constructed. Growth experiments in mineral medium (MM) supplemented with cholesterol revealed that the RHA1Δ*cyp125* strain was unable to grow on cholesterol (Table 1). To confirm that the observed phenotype was solely due to inactivation of *cyp125*, a complementation experiment was performed in which $cyp125_{RHA1}$ was supplied in *trans*. The complemented strain, RHA1Δ*cyp125*+pTip-QC1$cyp125_{RHA1}$, displayed a restored wild-type growth phenotype in MM supplemented with cholesterol (Table 1). Wild-type RHA1 and RHA1Δ*cyp125* were subsequently grown in mineral liquid media on a range of other sterols, steroids and their metabolites as sole carbon and energy sources (Table S1). RHA1 grew readily on all tested compounds. By contrast, RHA1Δ*cyp125* failed to grow on epicholesterol, 5α-cholestanol and on the plant sterol mixture β-sitosterol/β-sitostanol/campesterol. Remarkably, growth of strain RHA1Δ*cyp125* on 3-ketone oxidized derivatives of two of these sterols, 4-cholestene-3-one and 5α-cholestane-3-one, was unimpaired, likely due to degradation of the steroid ring structure (Table S1, Fig. 1). We thus conclude that CYP125 is essential for 3-hydroxy-sterol degradation. The phenotype of RHA1Δ*cyp125* was investigated further by growing the mutant in mineral liquid media supplemented with cholesterol and an additional non-repressing carbon source (i.e. pyruvate or glycerol). In contrast to the wild-type strain, RHA1Δ*cyp125* did not significantly transform cholesterol under these conditions (Fig. 2).

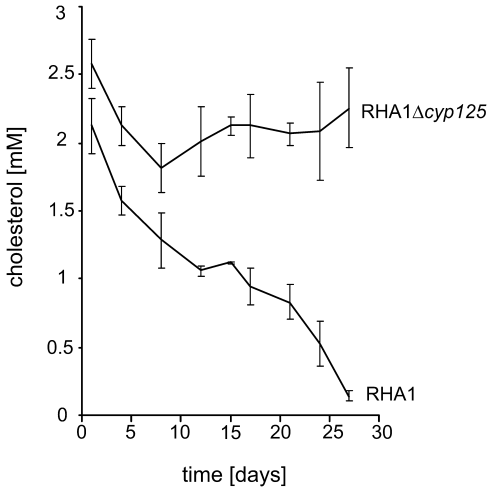


Fig. 2. Cholesterol degradation by cell cultures of strains RHA1 and RHA1Δ*cyp125* grown in mineral liquid media supplemented with pyruvate (20 mM) and cholesterol (2.5 mM). The data represent averages of triplicates. Error bars indicate standard deviations.

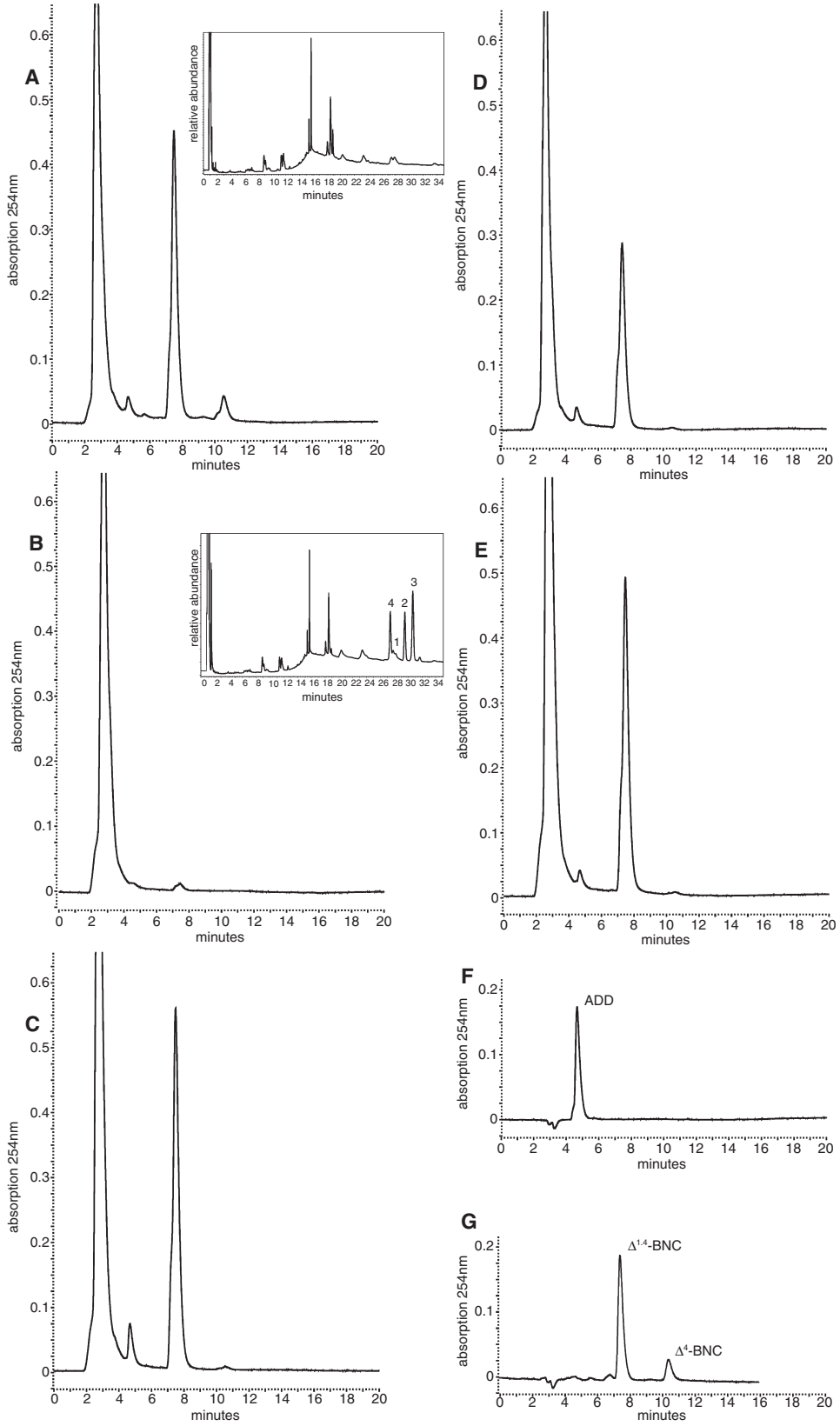
To further investigate the initial cholesterol-transforming enzymes of RHA1, we assayed pyruvate-grown cultures of wild-type RHA1 and mutant strain RHA1Δ*cyp125* that had been induced with cholesterol for total 3β-hydroxysteroid oxidation activity (Yang *et al*., 2007). These studies comprised assays for extracellular and intracellular activities arising from CHO and 3β-HSD. When cholesterol was used as a substrate in these assays, no activity was detected in either supernatants or cell lysates of these cultures, consistent with the lack of transformation of cholesterol by RHA1Δ*cyp125*. By contrast, 3β-hydroxysteroid oxidation activity was detected in lysates of cholesterol-induced cells of RHA1 (0.27 μM min^{-1} mg^{-1}) and RHA1Δ*cyp125* (0.76 μM min^{-1} mg^{-1}) when 5-pregnene-3β-ol-20-one was used as a substrate in the assay. Overall, these data indicate that CYP125 is essential for cholesterol degradation by RHA1, and that it catalyses an obligate first reaction in the cholesterol catabolic pathway.

CYP125 has a role in sterol side-chain degradation

We hypothesized that $CYP125_{RHA1}$ might have a specific role in sterol side-chain degradation. To substantiate this hypothesis, we used *R. rhodochrous* RG32, a mutant of *R. rhodochrous* DSM43269 which only degrades the side-chain of cholesterol, transforming it to ADD (Fig. 1, compound V) and $\Delta^{1,4}$-BNC (Fig. 1, compound IV) (Fig. 3A). First, we cloned *cyp125* from *R. rhodochrous* DSM43269 by screening a genomic library of this strain with degenerate PCR primers based on conserved amino acid sequences found in actinobacterial CYP125s. A positive clone, containing 8.7 kb of insert DNA, was obtained, sequenced and analysed. The insert carried $cyp125_{DSM43269}$, encoding a protein sharing 76% amino acid sequence identity with $CYP125_{RHA1}$ (Fig. S1). Moreover, the *cyp125* locus is similarly organized in *R. jostii* RHA1 and *R. rhodochrous* DSM43269. More specifically, the genes immediately downstream of *cyp125* in DSM43269 encode proteins sharing 56%, 74% and 86% amino acid sequence identity to those encoded by *ro04676*, *ro04677* and *ro04678*, respectively, in RHA1. Upstream of $cyp125_{DSM43269}$, orthologues of *ro04654* (82% identity) and *ro04653* (82% identity) were located, as well as genes encoding hypothetical proteins that have no counterparts in RHA1.

We then specifically disrupted *cyp125* in RG32, yielding mutant strain RG32Ω*cyp125*. Whole-cell biotransformations of 3-hydroxy-sterols by RG32Ω*cyp125* revealed that the mutant was blocked in the ability to degrade sterol side-chains (Fig. 3). Cell cultures of RG32Ω*cyp125* incubated with cholesterol showed no formation of ADD or $\Delta^{1,4}$-BNC (Fig. 3B). Similar results were obtained when RG32Ω*cyp125* cell cultures were incubated with 5α-cholestanol and β-sitosterol (data not shown). Contrary to RHA1Δ*cyp125*, cholesterol was rapidly converted by RG32Ω*cyp125* to 4-cholestene-3-one and 1,4-cholestadiene-3-one, which accumulated in the medium (Fig. 3B inset). Indeed, cholesterol-induced cells of RG32 and RG32Ω*cyp125* contained high levels of 3β-hydroxysteroid total oxidation activity using cholesterol as a substrate (0.34 and 0.73 μM min^{-1} mg^{-1} respectively). By contrast, no extracellular activity was detected in either strain. Reintroduction of $cyp125_{DSM43269}$ into RG32Ω*cyp125* under its native promoter fully restored the ability of the strain to degrade the cholesterol side-chain (Fig. 3C). This excludes the possibility that side-chain degradation in RG32Ω*cyp125* was blocked by polar effects rather than by disruption of *cyp125* directly.

Fig. 3. HPLC profiles of whole-cell biotransformations of cholesterol by cell cultures of (A) *R. rhodochrous* strain RG32 showing the formation of 1,4-androstadiene-3,17-dione (ADD) and 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid ($\Delta^{1,4}$-BNC) via selective sterol side-chain degradation, (B) mutant strain RG32Ω*cyp125* and (C) $cyp125_{DSM43269}$ complemented mutant strain RG32Ω*cyp125*. HPLC profiles of whole-cell biotransformations of 5-cholenic acid-3β-ol (D) and 5-cholestene-26-oic acid-3β-ol (E) by cell cultures of *R. rhodochrous* mutant strain RG32Ω*cyp125* are also shown. Profiles of authentic ADD (50 μM, F) and $\Delta^{1,4}$-BNC (G), obtained by incubating authentic 3-oxo-23,24-bisnorchol-4-en-22-oic acid (50 μM, Δ^4-BNC) with purified Δ1-KSTD1 (Knol *et al*., 2008), are included as reference samples. Insets: GC profiles showing the accumulation of 4-cholestene-3-one (2), 1,4-cholestadiene-3-one (3) and 5α-cholestane-3-one (4) from cholesterol (1) by *R. rhodochrous* mutant strain RG32Ω*cyp125*, but not strain RG32.



CYP125$_{DSM43269}$ is involved in formation of the sterol C26-oic acid intermediate

We then tested the ability of mutant strain RG32Ω*cyp125* to convert each of two predicted sterol side-chain degradation pathway intermediates: 5-cholestene-26-oic acid-3β-ol (Fig. 1, compound III) and the C24-oic intermediate 5-cholenic acid-3β-ol. Whole cell biotransformations performed with cultures of mutant strain RG32Ω*cyp125* resulted in conversion of both 5-cholenic acid-3β-ol and 5-cholestene-26-oic acid-3β-ol to ADD and $\Delta^{1,4}$-BNC (Fig. 3D and E). As predicted, RHA1Δ*cyp125* was able to grow on both of these compounds (Table S1). Both diastereomers of 5-cholestene-26-oic acid-3β-ol appeared to be metabolized, because 75 mol% of the added substrate was converted into ADD and $\Delta^{1,4}$-BNC. These results show that CYP125 is essential for the conversion of cholesterol into the C26-oic acid catabolite during sterol side-chain degradation by both RG32 and RHA1.

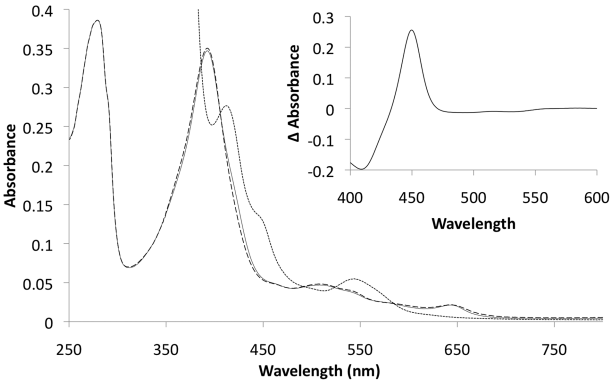


Fig. 4. The absorption spectrum of CYP125$_{RHA1}$ in the oxidized state as isolated (solid line) and incubated with 10 μM cholesterol in oxidized (dashed line) and reduced (dotted line) states. The inset shows the reduced CO-difference spectrum of the enzyme incubated with 10 μM cholesterol. The sample contained 2.9 μM purified CYP125$_{RHA1}$, 0.1 M potassium phosphate buffer, pH 7.0, 25°C; cholesterol was added from a 1 mM stock solubilized in 10% 2-hydroxypropyl-β-cyclodextrin.

Production and purification of CYP125$_{RHA1}$

To biochemically characterize CYP125$_{RHA1}$, we homologously produced and purified recombinant CYP125$_{RHA1}$ with a 6-histidine tag. Expression of *cyp125$_{RHA1}$* was first attempted in *Escherichia coli* BL21(DE3) using T7 promoter-based expression vectors and conditions known to promote expression of P450 proteins, such as the addition of δ-aminolevulinic acid, $FeCl_3$, trace elements and thiamine (Parikh *et al*., 1997; Keizers *et al*., 2004). However, CYP125$_{RHA1}$ was not produced in significant amounts in *E. coli*. By contrast, *cyp125$_{RHA1}$* was well expressed in *R. jostii* RHA1 using the pTip-QC1 vector (Nakashima and Tamura, 2004). Addition of δ-aminolevulinic acid and other additives, usually necessary to promote expression of properly folded and soluble P450 proteins in *E. coli*, was not needed for homologous production of CYP125$_{RHA1}$ in *R. jostii* RHA1.

CYP125$_{RHA1}$ was purified using Ni^{2+}-NTA affinity chromatography and was determined by SDS-PAGE analysis to be in excess of 95% pure. The CO-difference spectrum of purified CYP125$_{RHA1}$ displayed a maximum at 451 nm (Fig. 4, inset), indicating the haem iron thiolate ligation remained intact throughout the protein's purification. The absorption spectrum of the purified ferric CYP125$_{RHA1}$ had a maximum at 392 nm and a shoulder at 422 nm (Fig. 4). Based on analysis of this spectrum (see *Experimental procedures*), the preparation is estimated to contain ~93% high spin state haem iron.

Spectroscopic analysis of sterol binding

Spectroscopic assays were performed with purified CYP125$_{RHA1}$ to investigate its binding to sterols. Following the addition of cholesterol (Fig. 5A) or 5α-cholestanol (data not shown) in a solution of 10% 2-hydroxypropyl-β-cyclodextrin, CYP125$_{RHA1}$ exhibited a spectral change with a pronounced trough at 422 nm and a peak at 392 nm, consistent with the decrease in the low-spin character of the haem iron associated with substrate binding. The difference spectrum also exhibited a perturbation at 395 nm in comparison with the typical type I binding spectrum. A perturbation at the same wavelength was observed upon addition of 5-cholestene-26-oic acid-3β-ol in 10% 2-hydroxypropyl-β-cyclodextrin, although the acid elicited no underlying type I spectral change at concentrations up to 20 μM (Fig. 5A). Cholesterol also induced a type I binding spectrum when added in the presence of other solubilizing agents, such as Triton WR1339 and dimethylsulphoxide. However, the spectral shifts were much weaker than in the presence of 2-hydroxypropyl-β-cyclodextrin (data not shown).

Using Eq. 1, apparent K_D values for cholesterol, 5α-cholestane-3β-ol, and 4-cholestene-3-one were evaluated to be 0.20 ± 0.08 μM, 0.15 ± 0.03 μM and 0.20 ± 0.03 μM respectively. The concentrations of enzyme calculated using this equation (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration calculated using the extinction coefficient for the reduced CO-difference spectrum of $\varepsilon_{450-490}$ = 91 mM^{-1} cm^{-1} (3.7 μM), although this extinction coefficient has not been independently verified for this isozyme. The high quality fit of the equation to the binding data (Fig. 5B–D) supports a binding stoichiometry of 1:1 and suggests that CYP125$_{RHA1}$ does not harbour a ligand as isolated despite the proportion of high-spin iron. Finally, CYP125$_{RHA1}$ exhibited maxima at 451 nm in CO-difference spectra taken after

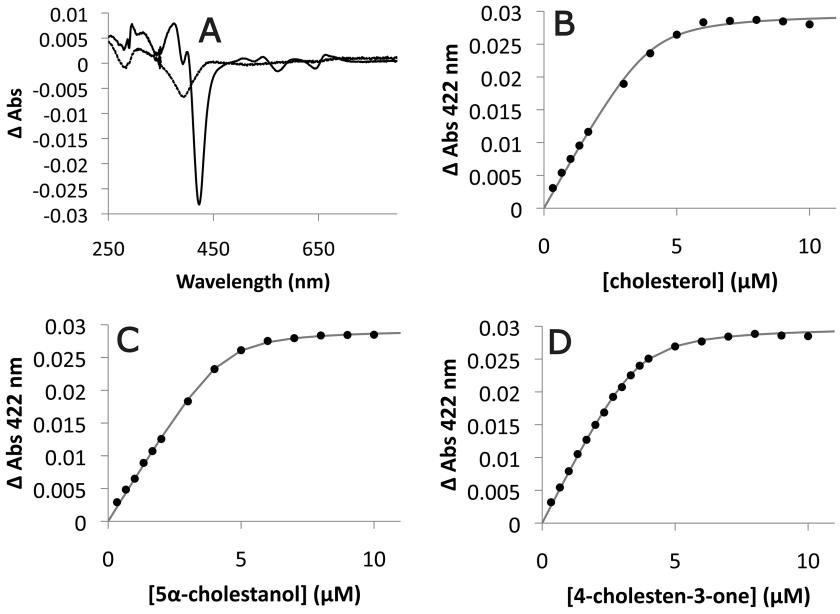


Fig. 5. Binding of steroids to purified CYP125$_{RHA1}$.
A. Spectral responses of 3.7 μM purified CYP125$_{RHA1}$ induced by 10 μM cholesterol (solid line) and 10 μM 5-cholestene-26-oic acid-3β-ol (dashed line). The dependence of the absorbance change of CYP125$_{RHA1}$ at 422 nm on (B) cholesterol, (C) 5α-cholestanol and (D) 4-cholestene-3-one concentration. The best fit of Eq. 1 to the data as determined using R is represented as a grey line with fitted parameters $K_D = 0.20 \pm 0.08$ μM, $\Delta A_{max} = 0.0298 \pm 0.0006$, and $[E] = 4.0 \pm 0.2$ μM for cholesterol; $K_D = 0.15 \pm 0.03$ μM, $\Delta A_{max} = 0.0293 \pm 0.0002$, and $[E] = 4.3 \pm 0.1$ μM for 5α-cholestanol; and $K_D = 0.20 \pm 0.03$ μM, $\Delta A_{max} = 0.0300 \pm 0.0002$, and $[E] = 3.6 \pm 0.1$ μM for 4-cholestene-3-one. Steroids were prepared as stock solutions in 10% 2-hydroxypropyl-β-cyclodextrin which alone did not induce a CYP125$_{RHA1}$ spectral response.

each binding experiment, indicating that the haem-thiolate ligation remained intact.

Discussion

The current study presents several lines of evidence identifying CYP125 as a steroid 26-monooxygenase that catalyses the initial step in microbial sterol side-chain degradation (Fig. 1). First, a *cyp125* deletion mutant of *R. jostii* RHA1 was unable to grow on or transform several 3-hydroxy-sterols with relatively long unactivated aliphatic side-chains. Second, a *cyp125* disruption mutant of *R. rhodochrous* RG32 was completely blocked in cholesterol side-chain degradation. However, this mutant was still able to degrade the side-chain of 5-cholestene-26-oic acid-3β-ol (Fig. 1, compound III), an expected intermediate of cholesterol side-chain degradation. Mutant RG32Ω*cyp125* thus is unable to form the sterol C26-oic acid intermediate, strongly indicating that CYP125$_{DSM43269}$ catalyses the oxidation of the sterol at C26. Finally, CYP125$_{RHA1}$ bound cholesterol, 5α-cholestane-3β-ol and 4-cholestene-3-one in a manner typical of P450 substrates: each compound induced a transition in the spin state of the haem iron and each bound with apparent submicromolar dissociation constants. The conclusion that CYP125 is a steroid 26-monooxygenase extends previous studies in which an NADH-dependent mixed function oxidase system was reported to be responsible for the first step in the mycobacterial sterol side-chain degradation pathway (Szentirmai, 1990) catalysing sterol C26-oxidation (Ambrus *et al.*, 1995).

Our data indicate that the oxidation of C26 is an essential first step of cholesterol degradation in *R. jostii* RHA1. The RHA1Δ*cyp125* mutant not only failed to detectably transform cholesterol, but grew on 3-oxo steroids, such as 4-cholestene-3-one and 5α-cholestane-3-one, as effectively as the wild-type strain. This indicates that in *R. jostii* RHA1, C26-oxidation precedes oxidation of the 3β-hydroxyl moiety (Fig. 1). RHA1Δ*cyp125* is likely able to grow on 3-oxo steroids by degrading steroid A and B rings, resulting in the formation of 2-hydroxyhexa-2,4-diene-oic acid that is further metabolized to form pyruvate and propionyl-CoA for growth (Fig. 1; van der Geize *et al.*, 2007). Previously, it was suggested that the microbial catabolism of cholesterol was initiated by ring oxidation (Sojo *et al.*, 1997; Chen *et al.*, 2006; Chiang *et al.*, 2008). Indeed, *R. rhodochrous* RG32Ω*cyp125* is capable of performing ring oxidation in the absence of CYP125, illustrating that the order of ring oxidation and sterol side-chain oxidation may vary between different species of bacteria. Consistent with the conclusion that CYP125$_{RHA1}$ initiates cholesterol degradation, genes encoding putative CHOs in RHA1 (*ro03863, ro04305*, *ro06201*) were not upregulated during growth on cholesterol and are located outside of the cholesterol catabolic gene cluster (McLeod *et al.*, 2006; Van der Geize *et al.*, 2007). Although 3β-HSD has not been definitively identified in RHA1, *ro04707* encodes a protein sharing 43% amino acid similarity with 3β-HSD of *M. tuberculosis* (Rv1106c) and is located proximal to the genes encoding the Mce4 steroid transporter (Mohn *et al.*, 2008). Indeed, *ro04707* was upregulated in cholesterol-grown RHA1 cells (Van der Geize *et al.*, 2007). While no cholesterol-transforming 3β-HSD activity was detected in RHA1, a 3β-HSD was expressed that transformed 5-pregnene-3β-ol-20-one, a 3β-hydroxysteroid with a shortened C21 side-chain. 3β-HSD$_{RHA1}$ thus appears to

have a high substrate specificity for side-chain-degraded cholesterol metabolites. This is similar to 3β-HSD of *M. tuberculosis* (Rv1106c) which had threefold higher activity towards 5-pregnene-3β-ol-20-one compared with cholesterol (Yang *et al*., 2007). It is possible that in RHA1, side-chain and ring degradation occur concurrently after C26 and C3-ol have been oxidized.

It is unclear whether CYP125 catalyses the oxidation of C26 to the corresponding carboxylic acid or only to an intermediate state. Various P450s have been reported to catalyse multistep oxidations (Helliwell *et al*., 1999; 2001; Ro *et al*., 2006), including a P450 from *Pseudomonas putida* PpG777 which catalyses two sequential oxidations of linalool, to 8-hydroxylinalool and 8-oxolinalool respectively (Ropp *et al*., 1993). It was proposed that a second oxygenation step results in a transient *gem*-diol adduct that spontaneously dehydrates to a more stable carbonyl compound (Ullah *et al*., 1990). Interestingly, $CYP125_{RHA1}$ displays significant amino acid sequence identity (32%) with linalool 8-monooxygenase and thus might well catalyse the complete oxidation of the aliphatic sterol side-chain into the sterol 26-oic acid intermediate via a similar mechanism. Attempts to reconstitute the activity of $CYP125_{RHA1}$ have so far proved unsuccessful despite using a variety of electron donors, including the spinach ferredoxin and ferredoxin-reductase electron transport chain and the peroxide shunt using cumene hydroxyperoxide (Hrycay *et al*., 1975). The physiological reductase of $CYP125_{RHA1}$ has not been identified yet.

Mycobacterium tuberculosis contains a CYP125 encoded by *rv3545c* located within the recently described *igr* operon (Chang *et al*., 2007; 2009). The bioinformatic data strongly suggest that the CYP125s of RHA1 and *M. tuberculosis* perform the same function: they are reciprocal best hits with 69% amino acid sequence identity that both occur in the cholesterol catabolic gene cluster (Van der Geize *et al*., 2007). However, the recently reported phenotype of an Δ*igr* mutant indicates that Rv3545c is not a steroid 26-hydroxylase (Chang *et al*., 2009): the mutant appeared to partially degrade cholesterol and transform the cholesterol labelled with ^{14}C at C26 into mycobacterial lipids. Additional studies are clearly required to definitively establish the role of CYP125 in *M. tuberculosis*. Indeed, while it is unclear if cholesterol degradation in mycobacteria occurs in the same manner as in *R. jostii* RHA1, two studies suggest that it does. First, *Mycobacterium* sp. NRRL B-3683, a mutant strain blocked in steroid ring degradation and able to selectively degrade the sterol side-chain, displayed a clear preference for substrates possessing a 3β-hydroxy-Δ5 ring configuration compared with the 3-keto-Δ4 configuration (Marsheck *et al*., 1972). Second, 3β-HSD of *M. tuberculosis* (Rv1106c) showed threefold higher activity towards 5-pregnene-3β-ol-20-one, a sterol with a C21 side-chain, compared with cholesterol, suggesting that sterols with shortened side-chains are preferred substrates of 3β-HSD (Yang *et al*., 2007). Regardless of the precise function of CYP125 in *M. tuberculosis*, its gene is upregulated during growth of *M. tuberculosis* in macrophages (Kendall *et al*., 2004) and $CYP125_{H37Rv}$ is more resistant to nitric oxide than other P450s of H37Rv (Ouellet *et al*., 2009). Moreover, the gene appears to be important for infection in mice (Chang *et al*., 2007; 2009). CYP125 may thus be an interesting target for the development of novel antituberculosis drugs.

Experimental procedures

Bacterial strains, plasmids and chemicals

Plasmids and bacterial strains used are listed in Table S2. 5-Cholestene-3β-ol, 5α-cholestane-3β-ol, 4-cholestene-3-one and 5-cholestene-24β-ethyl-3β-ol (75%) were obtained from Sigma-Aldrich. 5α-Cholestane-3-one was obtained from Acros Organics. 5-Pregnene-3β-ol-20-one was obtained from ICN Biomedicals. 5-Cholestene-3α-ol, 23,24-bisnor-5-cholenic acid-3β-ol, 5-cholenic acid 3β-ol and 1-(5α)-androstene-3,17-dione were obtained from Steraloids. 4-Androstene-3,17-dione and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostane-5-oic acid were provided by Schering-Plough (Oss, the Netherlands).

Construction of R. jostii *RHA1*Δcyp125

A *cyp125* unmarked single gene deletion mutant of *R. jostii* RHA1 was constructed using the *sacB* counter-selection system (Van der Geize *et al*., 2001). Genomic DNA of *R. jostii* RHA1 was isolated as described (Van der Geize *et al*., 2000). Mutagenic plasmid pDEL$cyp125_{RHA1}$ was constructed for *cyp125* deletion, as follows. The upstream region of *cyp125* was amplified by PCR using forward primer 5′-tcgac atccacttgatgaaggagaccg-3′ and reverse primer 5′-gcgACTAG Tcactgctgtctcctgccctaagc-3′, containing a SpeI restriction site (shown in capital letters). The resulting 1421 bp amplicon was cloned into SmaI-digested pK18*mobsacB*, resulting in pK18*mobsacB*UP*cyp125*. A 1451 bp amplicon of the *cyp125* downstream flanking region including the *cyp125* stop codon was obtained using forward primer 5′-cgcACTAGTtgacccctg attcagcggcgtcgg-3′ (SpeI restriction site) and reverse primer 5′-cgcAAGCTTgaacgaggacggcaagatcacgtccc-3′ (HindIII restriction site). This amplicon was digested with SpeI/HindIII and ligated into SpeI/HindIII linearized pK18*mobsacB* UP*cyp125*, resulting in pDEL$cyp125_{RHA1}$. Deletion of *cyp125* from RHA1 was confirmed by PCR using forward 5′-gcctcga cgattactggtgtgc-3′ and reverse primer 5′-cctcggacagaa ggagaacagc-3′.

Functional complementation of mutant strain RHA1Δ*cyp125* was performed by electrotransformation (Van der Geize *et al*., 2000) of RHA1Δ*cyp125* cells with expression plasmid pTip-QC1$cyp125_{RHA1}$ (see below).

Growth of R. jostii *RHA1 and mutant RHA1*Δcyp125 *strain on sterols/steroids*

Pre-cultures of wild-type strain RHA1 and mutant strain RHA1Δ*cyp125* were grown for 3 days at 30°C with shaking

(220 r.p.m.) in MM (Masai *et al.*, 1995) supplemented with pyruvate (20 mM) and used to inoculate MM liquid media (1:50) supplemented with various sterols/steroids (1 g l^{-1}; Table S1) as sole carbon and energy source. Biomass production of *R. jostii* RHA1 cell cultures incubated with cholesterol were quantified by total protein content determination of sonicated cells (10 cycles of 30 s at 8 μm) using the Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as protein standard.

Biotransformation of cholesterol by R. jostii *RHA1 and mutant RHA1Δ*cyp125

For biotransformation of cholesterol, precultures of RHA1 and RHA1Δ*cyp125* were grown in MM supplemented with pyruvate (20 mM) for 3 days at 30°C with shaking (220 r.p.m.). The precultures were used to inoculate MM liquid media (1:50) containing pyruvate (20 mM) and cholesterol (2.5 mM).

Determination of intracellular and extracellular total 3β-hydroxysteroid oxidation activity

Total 3β-hydroxysteroid oxidation activity was determined by high-performance liquid chromatography (HPLC) analysis essentially as described by Yang *et al.* (2007). Cell cultures of RHA1 and RHA1Δ*cyp125* were grown in MM supplemented with pyruvate (20 mM) for 3 days to an OD_{600} of 3. Cell cultures of RG32 and RG32Ω*cyp125* were grown overnight in Luria–Bertani (LB) medium. Grown cultures were induced for 16 h by adding 0.5 mM cholesterol from a 10 mM stock prepared in isopropanol. The cell cultures (50 ml) were pelleted and the resulting supernatants were filter-sterilized and used for assaying extracellular cholesterol oxidation. The cell pellets were washed two times with 50 mM phosphate buffer (pH 7) supplemented with 5% (v/v) isopropanol and resuspended in 2 ml of the same buffer. Cell lysates were prepared by bead-beating. Cell lysates were centrifuged to remove cell debris. The 3β-hydroxysteroid oxidation assay was performed in 100 mM triethanolamide hydrochloride buffer (pH 8.5) supplemented with 0.05% (v/v) Triton X-100, 3.5 mM NAD^+ and either 200 μM cholesterol or 200 μM 5-pregnene-3β-ol-20-one and incubated at 30°C for several hours (Yang *et al.*, 2007). 4-Cholestene-3-one and 4-progestene-3-one formation was quantified by HPLC-UV_{254nm} using calibration curves.

Steroid analysis

Steroid content of the cell cultures was analysed by HPLC and gas chromatography (GC). Culture samples (0.5 ml) were mixed with 2 ml of 80% methanol in water solution and filtered (0.2 μm) prior to analysis by HPLC-UV_{254nm}. HPLC was performed on an Alltima C18 column (250 × 4.6 mm; Alltech, Deerfield, USA, 35°C) using a mobile phase consisting of methanol : water (80:20) supplemented with 1% formic acid at a flow rate of 1 ml min^{-1}. For analysis of 4-cholestene-3-one and 1,4-cholestadiene-3-one a mobile phase consisting of acetonitrile : tetrahydrofuran (75:25) at a flow rate of 2 ml min^{-1} was used. Samples (0.5 ml) for GC analysis were mixed with 10% H_2SO_4 (10 μl) and ethyl acetate (2 ml) and the upper organic layer was subjected to GC. GC was performed on a (5% phenylo)-95% methoxypoly siloxane Heliflex AT-5 ms column (30 m × 0.25 mm, ID × 0.25 μm; Alltech, Deerfield, USA) with FID-40 detection at 300°C.

Production of $CYP125_{RHA1}$

The *$cyp125_{RHA1}$* gene was amplified by PCR on genomic DNA of RHA1 with forward primer 5′-CATATGgcgcagcccaat cttccagaggg-3′, containing an NdeI restriction site, and reverse primer 5′-GGATCCtcagtgtctgaccgggcaaccg-3′, containing a BamHI restriction site, such that the recombinant protein contains a 6-histidine tag. PCR was performed in a reaction mixture (25 μl) consisting of Tris-HCl (10 mM, pH 8), polymerase buffer, dNTP (0.2 mM), primers (0.8 μM) and Vent polymerase (0.1 U, New England Biolabs, Ipswich, MA) under the following conditions: 5 min 95°C, 30 cycles of 45 s 95°C, 45 s 65°C, 2 min 72°C, followed by 5 min at 72°C. A band of the expected size for *$cyp125_{RHA1}$* (1266 bp) was purified from agarose gel using GenElute Gel Extraction Kit (Sigma-Aldrich, Steinheim, Germany) and cloned into SmaI-digested pBlueScript KS(II) (Stratagene, La Jolla, CA, USA). The resulting plasmid was digested with NdeI and BamHI and the DNA fragment containing *$cyp125_{RHA1}$* was ligated into NdeI/BamHI-digested digested pTip-QC1.

$CYP125_{RHA1}$ was homologously produced in *R. jostii* RHA1 using expression plasmid pTip-QC1*$cyp125_{RHA1}$*. Cells were cultured in LB broth in the presence of 25 μg ml^{-1} chloramphenicol. *R. jostii* RHA1 cells were transformed with pTip-QC1*$cyp125_{RHA1}$* by electroporation and grown on LB-agar plates containing 25 μg ml^{-1} chloramphenicol for 2 days, after which a single colony was used to inoculate 50 ml of liquid medium which was incubated at 30°C (200 r.p.m.). When OD_{600} reached ~1.0 (~2–3 days) 2 l of medium inoculated with 20 ml of this preculture was incubated at 30°C. When the culture reached an OD_{600} of 0.6, thiostrepton was added to a final concentration of 50 μg ml^{-1} and the cells were incubated for a further 20 h before harvesting by centrifugation (4600 *g*, 4°C, 10 min) and subsequent washing with 0.1 M potassium phosphate buffer, pH 8.0. Cell pellets were flash frozen in liquid nitrogen and stored at –80°C until use.

Purification of $CYP125_{RHA1}$

The cell pellets were suspended in potassium phosphate buffer (pH 7.4) (Lussenburg *et al.*, 2005) containing DNase I (Roche diagnostics, IN). Cells were disrupted by bead beating and debris was removed by centrifugation at 10 000 *g* for 45 min at 4°C. The clear supernatant was passed through a syringe-driven 0.45 μm filter. Cell free extracts were loaded on a NTA column (Qiagen) equilibrated with 0.1 mM potassium phosphate, pH 7.4. The protein was washed with Buffer A containing 0.5 M NaCl and a brown fraction eluted with buffer A further supplemented with 50 mM L-histidine. The protein was exchanged into 0.1 M potassium phosphate, pH 7.4, concentrated to 20 mg ml^{-1}, flash frozen as beads in liquid nitrogen and stored at –80°C. P450 protein

concentrations were calculated from the reduced CO-bound difference spectrum using the extinction coefficient $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$ (Omura and Sato, 1964).

Spectroscopic analysis

UV-vis absorption spectra were recorded using a Cary 5000 spectrophotometer equipped with a thermojacketed cuvette holder (Varian, Walnut Creek, CA). The CO-bound form of $CYP125_{RHA1}$ was generated by first incubating samples with ~8 mM sodium dithionite for 10 min then slowly bubbling them with CO for 30 s. The proportion of purified protein containing high-spin ferric haem iron was estimated by comparing the spectra of $CYP125_{RHA1}$ to linear combinations of the spectra of $CYP125_{RHA1}$ in high and low spin states (Jung *et al.*, 1991; Jefcoate, 1978) generated by adding 0.5% Triton X-100 and 40% methanol, respectively, to the sample. The same values were obtained when using substrate-free cytochrome $P450_{cam}$ from *P. putida* as a low spin standard. Substrate-induced spectral responses were recorded in 0.1 mM KPi, pH 7.0 by titrating solutions of $CYP125_{RHA1}$ with 1.0 mM stock solutions of cholesterol, 5α-cholestane-3β-ol, and 4-cholesten-3-one in 10% 2-hydroxypropyl-β-cyclodextrin (Sigma). Equilibrium dissociation constants were calculated using Eq. 1.

$$\Delta A = \frac{[S]_T + [E]_T + K_D - \sqrt{([S]_T + [E]_T + K_D)^2 - 4[S]_T[E]_T}}{2[E]_T} \Delta A_{Max} \quad (1)$$

In this equation, ΔA is the change in absorbance observed in the sample, $[S]_T$ is the total ligand concentration, $[E]_T$ is the total enzyme concentration, K_D is the equilibrium dissociation constant, and ΔA_{Max} is the change in absorbance at infinite ligand concentration. A non-linear least-squares fit of the equation to the data was obtained using the program R (http://www.R-project.org).

Construction of R. rhodochrous *RG32Ω*cyp125

The *cyp125* orthologue in *R. rhodochrous* DSM43269 ($cyp125_{DSM43269}$) was identified using degenerate *cyp125* primers (forward 5′ (a/g)ac(a/c/g/t)gc(a/c/g/t)cc(a/c/g/t)at(a/c/t)tggtggaa and reverse 5′-gg(a/g)tt(c/t)tc(a/g)aa(a/c/g/t)gc(a/g)tc(c/t)tc(a/g)) based on the deduced amino acid sequences T^{33}APIWWN^{39} and D^{329}EDAFENP^{336} from $CYP125_{RHA1}$; these sequences are highly conserved in Nfa5180 and Rv3545c from *N. farcinica* IFM10152 and *M. tuberculosis* H37Rv respectively. A genomic library of *R. rhodochrous* DSM43269 in pRESQ (Petrusma *et al.*, 2009; Table S2) was screened by PCR using these degenerate primers. A single clone (pRESQ4679) was identified containing an 8.7 kb DNA insert. Nucleotide sequencing confirmed the presence of full-length (1254 bp) *cyp125* (DDBJ/EMBL/Genbank Accession No. FJ824698).

The *cyp125* gene was disrupted in *R. rhodochrous* strain RG32 essentially as described (Van der Geize *et al.*, 2000). An internal $cyp125_{DSM43269}$ fragment of 811 bp was amplified by PCR using forward primer 5′-gcacgaggaggtccgtgaggtc and reverse primer 5′-cgttgttggccgaggcgtacag and ligated into EcoRV-digested pK18*mobsacB*, yielding p*Ωcyp125*. This construct was used to transform *E. coli* S17-1 and was subsequently mobilized to mutant strain RG32 by conjugational transfer (Van der Geize *et al.*, 2001). Transconjugants were checked by PCR to confirm the *cyp125* gene disruption using forward primer 5′-acgcagccaccgatgacctgtt, annealing to a sequence upstream of $cyp125_{DSM43269}$, and reverse primer 5′-ctgcgtgcaatccatcttgttc, which is reverse complementary to part of the *aphII* gene of pK18*mobsacB*. A PCR product of the expected size (1903 bp) confirmed insertion of the disruption plasmid p*Ωcyp125* at the correct genomic locus.

*Functional complementation RG32Ω*cyp125

The intact *cyp125* DSM43269 gene and its flanking regions were isolated from DraIII/BspHI-digested pRESQ4679. A DNA fragment of 2.3 kb harbouring *cyp125* was treated with T4 DNA polymerase and blunt-ligated into EcoRV-digested shuttle vector pRRE1 (see below), resulting in pCOMP$cyp125_{DSM43269}$ that was used to transform electrocompetent cells of RG32*Ωcyp125* as described (Fernandes *et al.*, 2001). *E. coli-Rhodococcus* shuttle vector pRRE1 was constructed as follows. The *repA* and *repB* genes from *R. rhodochrous* DSM43269 endogenous plasmid pRC4 (GenBank/EMBL/DDJB accession number AB040101) were amplified from genomic DNA of strain DSM43269 using forward primer 5′-cgatggcaagccaccgcgaagc and reverse primer 5′-atcggacagaagctgactaagg. This amplicon (2.5 kb) was ligated into SmaI-digested pK18*mobsacB*. A 2.6 kb EcoRI/XbaI DNA fragment of the latter construct was subsequently treated with Klenow fragment and blunt-ligated into PsiI-digested pBs-Apra-ori (Van der Geize *et al.*, 2008), resulting in pRRE1.

*Whole-cell steroid biotransformations with RG32 and RG32Ω*cyp125

Cell cultures of parent strain *R. rhodochrous* RG32, mutant strain RG32*Ωcyp125* and the $cyp125_{DSM43269}$ complemented mutant strain were grown overnight in liquid LB medium, supplemented with kanamycin 25 μg ml^{-1} when appropriate, at 30°C with shaking (200 r.p.m.) until OD_{600} ~4 was reached. Sterols were added to the cell cultures at a final concentration of 0.5 mM from a 25 mM stock solution dissolved in acetone. Bioconversions were followed for 3 days of incubation at 30°C with shaking (200 r.p.m.). Accumulation of ADD and $\Delta^{1,4}$-BNC was analysed by HPLC-UV_{254nm} as described above in *Steroid analysis*.

Chemical synthesis of 5-cholestene-26-oic acid-3β-ol

Synthesis of 5-cholestene-26-oic acid -3β-ol was carried out using a modification of the method described by Williams *et al.* (2002) with diosgenin as starting material (Fig. S2). In the first step, the 3-hydroxy group was protected as a methyl ether, using NaH and MeI and a reaction time of 24 h. The resulting 3-methyl ether (product 1) was isolated in near 100% yield after precipitation from water. Next, the ether rings were reductively ring-opened under Clemmensen conditions by treatment with Zn/HCl in ethanol at reflux

temperature. After removal of the salts, extractive work up and a precipitation from acetone/water, the 16, 27-dihydroxylated product (product 2) was obtained in near 100% yield. A regio selective protection of the primary alcohol at C27 was carried out by reaction with *tert*-butyldimethylsilyl chloride and imidazole in N,N-dimethylformamide with 97% yield (product 3). For removal of the 16-hydroxy group, the Barton deoxygenation conditions were chosen. The C16-hydroxy group was transformed in the corresponding thiocarbonate with CS_2 under the influence of NaH. The intermediate thiocarbonate anion was quenched with methyl iodide. Next, a radical reduction reaction was carried out using Bu_3SnH and AIBN. After purification by silica gel column chromatography, the *tert*-butyldimethylsilyl-protected 3-methyl ether form of 27-hydroxycholesterol (product 4) could be isolated in near 100% yield with an estimated ^{1}H-NMR purity of > 80%. The *tert*-butyldimethylsilyl ether was removed under standard conditions using tetrabutylammonium fluoride in tetrahydrofuran, and silica gel column chromatography was used to purify product 5 with a yield of 73%. Oxidation to the 26-oic acid was carried out under Jones' condition, using a mixture of sulphuric acid and chromine trioxide. 5-Cholestene-26-oic acid-3β-ol-3-methyl ether (product 6) was obtained by column chromatography purification in 89% yield with an estimated ^{1}H-NMR purity of 80%. The final step in the synthesis was the removal of the 3-methyl ether by treatment with TFA in DCM at room temperature for 2 days. After aqueous work up, the trifluoroethanol ester was saponified with K_2CO_3 in methanol and purified by silica gel column chromatography, generating 5-cholestene-26-oic acid-3β-ol (product 7) in a low yield of 15% with a ^{1}H NMR purity of approximately 95% and consisting of a 1:1 mixture of diastereomers at C26. Apparently, during the strong acidic conditions used for the removal of the 3-methyl ether, enolization and protonation at C26 had occurred giving rise to a 1:1 mixture of stereo-isomers. The structure was confirmed by mass spectrometry (Fig. S3).

Acknowledgements

We are grateful to N. P. E. Vermeulen, J. Commandeur and B. van Vugt-Lussenburg (VU Amsterdam) for technical advice and support. We thank T. Tiemersma-Wegman (University of Groningen) for technical assistance in GC analyses. We thank T. Tamura for kindly providing pTip-QC1. This work was supported by grants from the Integration of Biosynthesis and Organic Synthesis program of Advanced Chemical Technologies for Sustainability and the Canadian Institutes for Health Research (to L.D.E.). J.C. is the recipient of studentships from NSERC of Canada and MSFHR.

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